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Review

Exosomes: Emerging Implementation of Nanotechnology for Detecting and Managing Novel Corona Virus- SARS-CoV-2

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Abstract

The spread of SARS-CoV-2 as an emerging novel coronavirus disease (COVID-19) had progressed as a worldwide pandemic since the end of 2019. COVID-19 affects firstly lungs tissues which are known for their very slow regeneration. Afterwards,

enormous cytokine stimulation occurs in the infected cells immediately after a lung infection which necessitates good management to save patients. Exosomes are extracellular vesicles of nanometric size released by reticulocytes on maturation and are known to mediate intercellular communications. The exosomal cargo serves as biomarkers in diagnosing various diseases; moreover, exosomes could be employed as nanocarriers in drug delivery systems. Exosomes look promising to combat the current pandemic since they contribute to the immune response against several viral pathogens. Many studies have proved the potential of using exosomes either as viral elements or host systems that acquire immune-stimulatory effects and could be used as a vaccine or drug delivery tool. It is essential to stop viral replication, prevent and reverse the massive storm of cytokine that worsens the infected patients' situations for the management of COVID-19. The main benefits of exosomes could be; no cells will be introduced, no chance of mutation, lack of immunogenicity and the damaged genetic material that could negatively affect the recipient is avoided. Additionally, it was found that exosomes are static with no ability for *in vivo* reproduction. The current review article discusses the possibilities of using exosomes for detecting novel coronavirus and summarizes state of the art concerning the clinical trials initiated for examining the use of COVID-19 specific T cells derived exosomes and mesenchymal stem cells derived exosomes in managing COVID-19.

Keywords: Exosomes; COVID-19; Specific T cell-derived exosomes; Mesenchymal stem cells-derived exosomes; Nanotechnology; SARS-CoV-2

1. Introduction

Past two decades, the Coronavirus had led to three consequential pandemics, Middle East Respiratory Syndrome (MERS), Severe Acute Respiratory Syndrome (SARS), and Coronavirus disease-2019 (COVID-19). After first reported case of novel

pneumonia in southern China's Guangdong province in November 2002, it was identified as SARS in February 2003, a respiratory viral infection due to SARS associated coronavirus (SARS-CoV or SARS-CoV-1) [1]. Later on, it affected more than 25 nations in Europe, North and South America, and Asia until it was ceased in July 2003 [2]. As per the World Health Organization (WHO), about 8 098 people worldwide were infected with SARS during the 2003 outbreak, out of which 774 died [3]. Saudi Arabia initially reported MERS coronavirus (MERS-CoV) in September 2012 [4]. This outbreak was then seen in the United Arab Emirates, Iran, and Republic of Korea between 2012 and 2017 [5]. As per the WHO data, MERS infected 2494 and killed 858 people worldwide from 2012 to the end of November 2019 [6]. Now, the world, since the end of 2019, is facing the transmission of the 2019 novel coronavirus (SARS-CoV-2), causing COVID-19. Actually, COVID-19 affected more than 200 countries globally (Fig. 1). WHO confirmed 200 840 180 cases of COVID-19 which included 4 265 903 deaths worldwide from 30 December 2019 till 5th August 2021 [7].

Exosomes look promising to combat these pandemics since they play a crucial role in the immune system response against several viral pathogens. Further, the cargo of both viral and host factors boosts or hinders the associated immune response *via* diverse mechanisms. Also, due to the inherent potential of exosomes in transferring materials between cells, they can be employed as natural drug delivery vehicles. Many studies proved the potential of exosomes as viral elements or host factors delivery vehicles to acquire immune-stimulatory effects to be utilized as a vaccine or drug delivery tool. In this context, currently available literature on exosomes and their role in treating viral infections such as COVID-19 will be reviewed herein.

In multicellular organisms, eukaryotic cells execute intercellular communications in order to maintain homeostasis and cell development. The local cell communications are achieved by direct cytoplasmic connection through the cell junctions, and the distant communications are established through the chemical messengers such as hormones, growth factors, cytokines, and other secreted molecules [8]. In recent times, researchers discovered extracellular vesicles (EVs) as mediators of intercellular communications with proved pathways [9]. In most eukaryotic cells, the multivesicular body (MVB) or endosomes are produced in the endosomal compartment fused with the cell plasma membrane, and released as lipid bilayer entities called EVs [10]. These multi-functional EVs are ubiquitously found in all types of biofluids which initially reported removing cellular waste. However, now we know these EVs cargo lipids, proteins, genetic materials such as messenger RNA (mRNA), small non-coding RNAs, and genomic DNA (gDNA) from mother cells to distant tissue cells and thereby transfer the cellular information [11]. Classification EVs can be based upon their cell morphology, size, biological function, origin, and the content they carry. They are more specifically categorised into two distinct classes based on their cellular origin or biogenesis, i.e. exosomes and microvesicles [12].

As aforementioned, EVs are formed either by the development of the cell membrane or within the lumen of the MVBs, and in this case, they are called intraluminal vesicles (ILVs). The other type is known as microvesicles or ectosomes and, in the latter, a fusion of MVBs along with cell membrane (exocytosis) to deliver ILVs, which is then stated as exosomes [11, 12]. The size of microvesicles ranges from 50 to 1000 nm extensively studied due to their role in blood coagulation [13]. However, they carry specific proteins as well as lipids to the designated receiver cell and are a crucial part of intercellular communication [14]. The diameter of exosomes,

nano-sized membrane vesicles released by reticulocytes on maturation, ranges from 30 to 100 nm [15]. In other words, during the formation of MVBs, the endosomal membrane was budding to the inside forming exosomes. These ILVs are exuded upon exocytosis (merging of MVBs with the plasma membrane). Johnstone with his team first described exosomes in the 1970s [16], and interest in these vesicles grown in recent years owing to their vast array of intriguing beneficial potential such as exosomal cargo as biomarkers in the diagnosis of various diseases and exosomes as nanocarriers in drugs delivery systems [17].

2. SARS-CoV-2

SARS-CoV-2, a novel human-infecting Beta coronavirus, belongs to the family Coronaviridae inherently different from SARS-CoV (79% similarity) and MERS-CoV [18]. Amino acid sequence in SARS-CoV-2 varies from other coronaviruses exclusively in the areas of 1ab polyprotein as well as S-protein or surface glycoprotein. The host receptor is directly attached to one of the subunits of the S-protein, facilitating the entry of viruses into the cells [19]. In SARS-CoV-2, the RNA binding domain of the S-protein has a stronger resemblance to SARS-CoV. It has been reported that the COVID-19 human receptor is an angiotensin-converting enzyme (ACE2). Coronaviruses, which includes SARS-CoV-2, gain access (ACE2) into human cells [20].

Structurally (Fig. 2), SARS-CoV-2 is an enclosed, non-segmented, pleomorphic or spherical shaped, single-stranded RNA virus, ranging from 150 to 160 nm in size. SARS-CoV-2 genome encodes approximately 25 proteins, notably, glycoproteins for instance, spike (S), envelop (E), nucleocapsid (N), and membrane (M) protein. Further, COVID-19 encodes a supplementary glycoprotein that has acetyl esterase and hemagglutination properties [21]. N protein, the basic structural unit of SARS-

CoV-2, encapsulates the RNA genome. It has been demonstrated that nucleocapsid is involved in the viral genome-related processes, viral replication, and host cell response to the viral infection. It has highly phosphorylated and prone to structural modifications, improving the affinity for viral RNA [22, 23]. There are possibilities of cross-reactivity of antibodies produced against N protein of SARS-CoV with SARS-CoV-2, but these heterophile antibodies of the SARS-CoV might not deliver cross-protection to SARS-CoV-2. However, they may be used for diagnostic purposes. N protein of SARS-CoV can counteract the immune response of the host by acting as a viral suppressor protein of RNAi (VSR). Infection occurs as VSRs repress RNAi at a pre-dicer and post-dicer level to counteract the host's defence mechanism.

SARS-CoV and SARS-CoV-2 had around 90% same sequence identity based on analysis of clustal W of N-protein of by NCBI amino acid blast. Therefore, the N-protein of SARS-CoV-2 might similarly act as VSR to SARS-CoV to counteract the host defense mechanism [24, 25].

The M protein determines the virus envelop, shape and affinity to bind all other structural proteins. M protein binding to N Protein provides the stable N protein-RNA complex and completes the viral assembly in the internal virion. E protein is the minor structural protein of SARS-CoV-2 which plays an important role in viral production as well as maturation [26]. Additionally, a transmembrane protein called S glycoprotein is present in the external portion of the virus, with a molecular weight of approximately 15 kDa. These S proteins help the virus bind the specific receptors, especially ACE2 of lower respiratory tract cells, by forming homotrimers protruding in the viral surface and thus entering the human cells/ host cell, which is necessary for the survival of the enveloped viruses such as SARS-CoV-2 [27]. The S protein further gets split into S1 and S2 subunits by the host cell protease, out of which S1 will

involve in host cell target, receptor binding, and cellular tropism, and the S2 will mediate the virus fusion in transmitting host cells [28].

3. Biogenesis and architecture of exosomes

Exosomal biogenesis occurs *via* the cell trafficking system in two pathways named the endosomal sorting complex needed for the transport (ESCRT) dependent and transport-independent pathways. This ESCRT, formed of four protein subunits (ESCRT 0 to IV), is considered central molecular machinery involved in forming exosomes from endosomes. ESCRT is active in local membrane remodelling in autophagy, cytokinesis, and viral budding and thus facilitates the formation of ILVs inside MVBs by stepwise action with Alg-2-interacting protein-X (Alix) and arrestin domain comprising 1 (ARRDC1), which are well known as associated proteins [29, 30]. During the formation of exosome, accumulation of proteins, lipids, and nucleic acids occurs at the cytosolic face of endocytic membrane microdomains with the aid of these associated proteins. Following these accumulations, an inward curvature occurs, then-budding of the microdomains occurs with cleaving and release of individual ILVs within the lumen of the MVB [31]. Further, ESCRT also facilitates the deubiquitination of sorted proteins within ILVs by interacting with e protein tyrosine phosphatase HD-PTP essential for the exosome functioning. Additionally, the inner curvature and the development of endocytic membrane microdomains were assisted by sphingomyelin-derivative ceramide; then the small GTPase Ral confer in the process of release of exosome through merging of MVBs along with plasma membrane [11, 32].

In the ESCRT dependent pathway, biogenesis of exosomes occurs successively, and it is a multistep event. It involves identifying cargo by ESCRT-0, and then the cargo is sorted into the nascent ILVs. Further, invagination of MVB's membrane takes place

with the aid of ESCRT-0, -I and -II. Vesicle maturation was then followed by this step and ends up due to mediation by ESCRT-III, which causes neck contraction, and finally, membrane scission is facilitated by vacuolar ATPase Vps4 and ILVs formation. Within exosomes, exosomal markers are released, namely, Alix, which is an additional protein, and Tsg101 that is a component of ESCRT-I [30, 33-35]. ESCRT-independent pathways also produce exosomes. Ceramide, a cone-shaped lipid, was found to mediate exosome biogenesis. Independent pathway, ESCRT is activated by lipids for example cholesterol and ceramides. Ceramide was found to have a significant role in the development of MVBs. Nanovesicles of 40-150 nm size are released by docking these MVBs with the aid of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) complexes along with the dominant plasma membrane. Ceramide is produced from the sphingomyelin present on the endosomal's membrane surface in the presence of neutral sphingomyelinase (nSMase). ILVs are produced after an impulsive inward curvature on MVB's membrane mediated by Ceramaides [36]. Further, tetraspanins such as CD81, CD9, CD63, and other molecules like syndecan- syntenin-ALIX complex, Tsg101, VCAM-1, phosphatidic acid, and α4 integrin had been found to have a critical part in the biogenesis and loading of exosome [30, 37, 38]. T cells slightly differ from this pathway as they produce EVs out of the cell's surface, which possesses attributes of exosomes, likely by utilizing the cell components including mechanisms at the cell membrane commonly allied with the endosomal biogenesis of ILVs [39]. Fig. 3 represents the biogenesis and structure of exosome.

Exosomes are composed of specially sorted proteins, nucleic acids, lipids and other contents majorly dependent on their site of origin. The exosome membrane lipid component is characterised by cholesterol and sphingomyelin in vital concentration

and ceramide that has a vital role in the biogenesis of ILVs [40]. Furthermore, in contrast with the normal cell membrane, exosome membrane lipids contain lysobisphosphatidic acid that facilitates cholesterol accumulation [41]. Additionally, exosome membrane proteins include tetraspanins clusters and other transmembrane proteins. These tetraspanins have a role in luminal cargo loading via interaction with cytosolic proteins [42]. Additional tetraspanins act independently and are involved in the surface and intracellular trapping of signalling proteins such as b-catenin, E-cadherin, and Wnt [43]. Exosome membrane proteins also include the adhesion proteins as L1CAM (L1 cell adhesion molecule) and LAMP2 (lysosomal associated membrane protein 2); PGRL (CD81 regulatory-like protein), flotillin, and stomatin which bind lipids; the enzyme alanyl aminopeptidase N [43]; insoluble fibronectin, a surface glycoprotein [44] and integrins (Fig. 3).

Furthermore, the cargo proteins in the exosome include cytoskeletal and allied proteins like vimentin, actin, annexin, and talin. It also contains protein structure and function guarding units called chaperones such as Hsp 70, Hsp 90 and HSc 70. In addition, Exosome cargo Enzymes include glyceraldehyde3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase 1 (PGK1). Moreover, luminal proteins, various GTPase such as Arf6, RNAs such as miRNAs, RNA binding proteins, proteins that modulate RNA functions such as Y-box binding protein 1 and Argonaute 2 (AGO2), and also non-coding RNAs and DNA sequences are included in the exosomes cargo [45-49].

4. Exosomes in viral diseases

To survive, viruses usually evolve some mechanisms for facing the host immune system. Nevertheless, pathogens have a counteracting mechanism for each process

performed by the immune system [50, 51]. Exosomes are usually injected by viral components where these viral antigens can enhance their survival *via* decoying the immune system, cloaking viral genomes [52, 53]. Moreover, exosomes can act as biomarkers since they carry the viral antigen, which can be used for targeted therapy and biomarkers [54]. The immune response is regulated in viral infections by the viral and host components released, including exosomes and some EVs. Exosomes are implicated in the pathogenesis of various viruses. For instance, in the case of HIV, EVs and exosomes were imprisoned by cells then viral, and host proteins and RNA contribute to the replication and infection of viral components in the recipient [55]. $CD4^+$ as well as $CD8^+$ T-cell activation, are inhibited by the released exosomes. Table 1 shows the role of exosomes in facilitating different examples of viral infections. The release of exosomes by virally infected cells can promote the immune response against viruses by activating different mechanisms. Some examples are enumerated in Table 2.

5. Exosomes in COVID-19

As aforementioned, EVs from almost all cell kinds lead to intercellular transmission by conveying biological entities such as lipids, proteins and nucleic acids to beneficiary cells. The cells infected with the virus release exosomes and these exosomes enable infection by transferring viral-derived miRNAs and proteins that are nothing but viral components. In addition, exosomes possess receptors for viruses which makes the recipient cell more prone to virus invasion. Earnest et al., [81] reported that tetraspanin CD9 and TMPRSS2 were found to ease the entry of MERS-coronavirus and active lung infection of mouse *in vivo*. Exosomes and microvesicles contain CD9 molecules within them, and these molecules play a vital role in the exosome biogenesis and the loading of cargo into exosomes. Exosomes derived from

infected cells transfer CD9 molecules which might contribute to enhance virus entry. On uptake of exosomes, the delivery of exosomal cargo to recipient cells occurs and thus promotes the susceptibility of the recipient cell for virus infection. Besides, it has been confirmed that CD9 molecules participate in the entry of the protein-protein network in the MVBs membrane within exosomes [82] and thus favourably affect the loading of COVID-19 viral proteins. It was found that there is an increase in circulating exosomes that contain lung-associated self-antigens, viral antigens as well as 20S proteasome in coronavirus infections. This evidence supports the idea that the SARS-Cov-2 virus-infected cells yield exosomes containing viral proteins [83]. The greater affinity of SARS-Cov-2 virus to human ACE2 than SARS-CoV was reported by Wan et al. [84], which augment the spread of COVID-19. Recently it has been confirmed that cleavage of spike protein by TMPRSS2 is required for the SARS-Cov-2 virus to move in and cause infection through interaction with ACE2 receptor. It has been evident that exosomes transfer ACE2 to recipient cells, providing a supporting function for the internalization and infection by the SARS-Cov-2. Following the sorting of ACE2 into exosomes, the SARS-Cov-2 virus enters the cells through internalization passage and its components including miRNAs as well as proteins may get similarly incorporated into exosomes [85]. Coronavirus internalization occurs *via* caveolin-1 dependent endocytosis followed by shedding of viral components from the cell membrane by the vesicles *via* a mechanism depending on dynamin supporting the idea of exosomes' role in spreading viral infections. Nevertheless, humoral as well as cellular response of the host can be triggered by exosomes derived from infected cells *via* the transfer of viral and self-antigens [86]. A549 lung epithelial cells on transduction with lentivirus overexpressing genes found in SARS-CoV-2 had led to further isolation of exosomes from A549 cells in the supernatant layer. These

exosomes were found to possess the viral genome. Both Exosomes and viral genes were held by human- induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), this was confirmed by detecting them both in cardiac cells. Besides, the increase of inflammation-related genes in hiPSC-CMs was caused by the uptake of exosomes which contained viral genes. These outcomes suggest that cardiomyocytes can hold SARS-CoV-2 RNA-containing exosomes and produce severe cardiac problems without direct viral infection [87, 88].

6. Exosomes as a source of diagnostic markers

Exosomes can be utilized as biomarkers since they are found to be present in almost all of our body fluids such as, urine, saliva, semen and lung lavage fluid. Their different composition in both normal and disease state like cancer will eventually aid in detecting abnormal body conditions [89].

In the diagnosis of infectious diseases, not much research was done on exosomes, and this area of research shall be of interest in the future. Literature suggested that the bronchoalveolar lavage fluid obtained from M bovis BCG–infected mice had exosomes containing mycobacterial proteins [90]. Additionally, the serum of patient suffering from tuberculosis (TB) had several mycobacterial proteins involved in the exosomes highlighting their importance as a biomarker for TB. Moreover, mycobacterial RNA was found to be released from infected macrophages with TB, which ensure the possibility of using this RNA as a biomarker for active TB detection [91]. Welker et al. [92] found that CD81 (exosomal protein) levels augmented in the serum of hepatitis C chronic patients. Both the level of this exosomal protein and other markers of liver inflammations like ALT were found to be positively correlated. Therefore, detecting the level of CD81 in exosomes of these patients might help in both the diagnosis and follow up of their diseased state [92].

7. Purification and characterization of Exosomes

Various viruses, including SARS-CoV-2, exploit or need the ESCRT pathway for entering the recipient cells. The extracted exosomes out of virus-infected cells are challenging to characterise and investigate as these vesicles possess a similar density and size range as that of the virus, which renders their separation very challenging. As an example, exosomes isolated from HCV or HIV-infected cells possess the same densities and sedimentation velocities as these viruses and cannot be readily isolated away from them. The sequential sucrose-gradient ultracentrifugation technique is considered the conventional method for exosomes' isolation from both body fluids and culture media [93]. Other approaches include; technologies based on microfiltration, microfluidic techniques, using precipitation reagents like Total Exosome Isolation reagent (Life Technologies Grand Island, USA) and ExoQuick™ (System Biosciences, Mountain View, USA), also SA, in addition to, using antibody-coated magnetic bead-based immunopurification. Different viruses, either enveloped or non-enveloped, can be purified similarly by alternating centrifugation and ultracentrifugation methods [93-95]. For the characterization and detection of exosomes when present with the virus, many methods have been utilized, which include size analysis using NanoSight tracking analysis system for nanoparticles, detection by electron microscopy examination, and by using immunoblot analysis for detecting the presence of exosome protein markers like CD81, CD63, Annexin5, ICAM1, TSG101, Alix and FLOT1 [94, 96]. Unfortunately, certain viruses contain some exosomal proteins, so the marker is crucial for characterization by immunoblot analysis. Other analysis can present data about the presence of these exosomal proteins in viruses; for instance, proteomic studies by the use of liquid chromatography and tandem mass spectrometry (LC-MS/MS) reported that influenza

virus contains exosomal markers such as Annexin A3, ICAM1, CD81 and CD9, however, Alix and CD63 were not present [97]. Likewise, exosomes as well as retroviruses contain common molecules such as MHC-II, co-stimulatory molecules (CD28, CD54), integrins (CD11a, CD18) and complement neutralizing molecules (CD55, CD59) [98]. Therefore, the exosomes isolated by precipitation reagent or ultracentrifugation shall be essentially subjected to process of immunopurification such as CD63 immunomagnetic bead isolation or other efficient virus purification methods to acquire contamination-free exosomes. Further, exosomes were isolated from the serum of lung transplant beneficiaries diagnosed with respiratory viral infections by ultracentrifugation and the purity was estimated by means of a sucrose cushion [99]. The occurrence of lung self-antigens, 20S proteasome and a viral antigen for coronavirus were determined using immunoblot [99, 100].

The classical production methodology for exosomes involves performing differential centrifugation to the supernatants of the cell-culture medium. The successive centrifugation aim at removing the unwanted debris and dead cells. Firstly, the centrifugation process yields the final form of exosomes, this step usually lasts for about 1 h, and the centrifuge apparatus operated at 100 000 rpm. This step is followed by a purification step using ultracentrifugation to remove the nucleosomal fragments that might be released from apoptotic cells and any protein aggregates. The final obtained exosomes after all of the differential centrifugation steps were found to be homogenous. Furthermore, their homogeneity was tested in some researches by examination using transmission electron microscopy. However, this method is time-consuming because it is a multi-step procedure. Moreover, the percentage of exosome recovery ranged from 5%-25% [101].

Another method for producing exosomes is also adopted. The technique used in this method is based on the use of antibody-coated magnetic beads and flow cytometry. First, a coat of monoclonal antibodies for the molecules rich in exosomes is applied to the magnetic beads. This step is followed by the incubation step of beads with culture supernatants [102].

A third method was also used for exosomes production, which is more rapid than the two previous methods. The purification in this method comprises two steps; the first one involves using a 500-kDa membrane for ultrafiltration of the supernatant, and the second step using 30% sucrose/deuterium oxide (98%) cushion. This method is characterized by higher efficiency in removing unwanted proteins from exosomes and much higher yields of 40%-50% compared to the previously discussed classical methods [103,104].

8. Exosomal state of the art function in vaccination

The use of exosomes as vaccines has been originated from the cancer field. Numerous advantages have been detected by employing exosomes as vaccines against pathogens; (i) enhanced stability in terms of conformational environments for proteins; (ii) amplified molecular distribution owing to exosomal circulatation into body fluids enabling them to reach distal organs; (iii) extremely coherent correlation with antigen-presenting cells, owing to the expression of adhesion molecules on exosomal surface; (iv) more protection of nucleic acids and proteins against DNase, RNase and proteinases thereby providing a stable environment; and (v) exosomes can act as cross-priming since they are considered as one amongst the body's natural mechanism for antigen transport amongst cells [64]. It was found that CD4 T-cell clones can be triggered by the EVs released by B-cell lines bearing MHC class-II, adhesion and co-stimulatory molecules [105, 106]. Further, tumor peptide-pulsed

dendritic cells (DCs) exosomes were used as a vaccine for mice, they carried tumor-specific cytotoxic T lymphocytes (CTLs), and they successfully inhibited the growth of tumor in a T-cell dependent manner [106, 107]. Moreover, cytomegalovirus released exosomes carrying peptides, where influenza virus and Epstein -Barr virus have been found to directly stimulate the secretion of IFNγ *in vitro* by the human peripheral CD8 T-cells and possibly memory T-cells [108, 109]. In a study by Montaner-Tarbes et al., exosomes carrying antigens for porcine respiratory and reproductive syndrome virus (PRRSV) were detected in the serum of viremic (V) as well as non-viremic (NV) pigs. It was suggested that a vaccine against PRRSV could be developed as a novel formulation containing serum-derived exosomes contained antigenic viral protein free of the virus [110]. Size exclusion chromatography was used to isolate exosomes followed by their characterization as naive control (CN) pigs (PRRSV negative), V (PRRSV RNA positive and seropositive) pigs and NV (PRRSV RNA negative and seropositive) pigs. Furthermore, the antigenic properties of viral proteins derived from exosome were tested and established that the immune sera of pigs earlier exposed to PRRSV explicitly reacted with the exosomes from NV animals. It was found that there was a close similarity between the exosomal mediated antigenic activity and the antigenic activity enclosed in the vaccine available commercially. An important point to be considered for developing novel vaccines is that exosomes carrying viral antigens circulated in host serum without pathogen load detected in the peripheral circulation (NV) [110, 111].

Exosomes permit intercellular communication, and presenting antigens could induce a robust immune response. The antigen-presenting exosomes could be used as a novel strategy by modifying the exosomes to present viral antigens that would trigger high and specific CD8+ T cells also known as killer T cells as well as triggering B cell

reactions. EV-based vaccines such as expression of the spike protein of SARS-CoV-2 on an exosomal surface or delivering viral protein mRNAs via EVs are being developed against COVID-19 by some biotech companies [112].

Capricor Therapeutics is acting on two distinct EV-based vaccines which could induce a long-term protecting immune response against SARS-CoV-2 [113]. Firstly, an EV display vaccine, made up of the human HEK293 cells transfected with the vectors expressing the Spike, Nucleocapsid, Membrane and Envelope proteins, are the four structural proteins of SARS-CoV-2. It was previously established that developing a vaccine consisting of multiple forms of protein permits for the magnitude modulation in addition to the nature of the immune response involved in cytokine production and Th1 or Th2 stimulation [114]. The second type of EV contains five different mRNAs that encode for Spike, Nucleocapsid, Membrane, and Envelope proteins (LSNME) of modified SARS-CoV-2 and the full-length spike of Wuhan-1 isolate (Sw1). The vaccines were injected intramuscularly into mice and showed an immunity that lasted up to 2 months after the second booster dose. Finally, there were no vaccine-induced adverse reactions in mice, such as inflammation at the injection site, altered organ morphology, blood cell profiles, or body growth [115]. The Ciloa Company has come up with CoVEVax, a vaccine against COVID-19 based on HEK293T-derived CD81+/CD63+/CD9+ EVs. Due to merging with the patented EV-sorting peptide CilPP, the EVs are modified in such a way to display the entire S protein on their surface. Mice were subcutaneously given without adjuvants both the two components and vaccine holding DNA vector for the engineered EVs, and HEK293T-derived engineered EVs. Both humoral and cellular response was evoked only by this combination, which specific IgG measured to S1 or S2 peptide levels and antigen-specific IFN-production [116]. The biotech firm, Codiak BioSciences, is

developing a vaccine for COVID-19 called exoVACCTM. It is an advanced system of vaccine that takes advantage of the characteristic properties of EV, where it is possible to simultaneously deliver specific antigens besides immunostimulatory adjuvants to the antigen-presenting cells (APCs) to stimulate both innate as well as humoral immune response. On the other hand, exoVACCTM requires further research as there are possibilities of formation of various combinations of SARS-CoV-2 antigens and adjuvants, and in addition, their efficacy as well as specificity in vitro and animal models ought to be evaluated [117,118].

9. Can Exosomes be used in managing COVID-19?

Exosomes possess immunogenic activity in managing SARS coronavirus infection, and this activity has been reported in some studies. Kuate et al. had discussed using exosomes holding Spike (S) protein of SARS coronavirus in inducing the neutralizing antibody titers. In this study, a vaccine was developed where exosomes loaded with S protein SARS coronavirus, and the transmembrane domains of SARS-S are substituted by the G protein of vesicular stomatitis virus produced chimeric protein (SGTM) comprising exosomes [119]. The exosomes derived from SARS-CoV-2 virus-infected cells can prompt immune cell response and deliver therapeutic agents as they comprise specific targeting molecules, ACE2. Moreover, in the same context, drugs can be loaded into exosomes to limit the virus spreadability and replication in host cells. Exosomes are considered to be superior to other nano-delivery systems. For example, exosomes originate from cells, and hence they are safer and possess constant property unlike the other delivery systems such as liposomes [120]. It was found that exosomes derived from the virus-infected cells contributed to promoting virus infection as well as suppressed immune cell responses (Table 1 and 2). Therefore

inhibiting the exosome uptake by the neighbouring cells may be used as a strategy to overcome virus spreading.

Stem cells showed recently a crucial therapeutic role in regenerative medicine, where, mesenchymal stem cells (MSCs) therapy has moved from preclinical trials to clinical trials for managing various diseases [121-124]. For example, MSCs could be used in managing COVID-19 since they can fight inflammation; they were reported to diminish the pathological deteriorations in the lungs significantly and were found to inhibit the cell-mediated immune-inflammatory response in an animal model that was induced by the influenza virus [124]. When COVID-19 virus replicates in the infected patient's body, it induces a series of inflammatory responses that worsen the disease condition. This includes progressive damage of alveolar epithelial and capillary endothelial cells, which causes diffuse interstitial and alveolar oedema and ultimately leads to acute hypoxic respiratory insufficiency. So fighting inflammation will aid in managing severely affected patients with COVID-19, help re-establishment lung cells function, and establish lung tissue regeneration by using MSCs. Additionally, MSCs can act as an immunomodulator and having pro-angiogenic, anti-fibrotic and regenerative abilities [125].

Based on this fact, recently, a study was recorded with an identified number that focused to investigate the aerosol inhalation of exosomes derived from allogeneic MSCs in the management of COVID-19 patients with severe pneumonia [126]. It is known that MSCs derived exosomes (MSCs-Exo) are useful in the treatment of various diseases and are well known to suppress inflammation of the lungs and the pathological damage caused due to several kinds of lung injury. Almost certainly, exosome treatment could be a valuable tool for managing or at least inhibiting the spread of COVID-19 in the host, yet, further study is necessary. In this regard, some

in-progress clinical trials on the MSCs or MSCs-Exo for the COVID-19 treatment are listed in Table 3.

MSCs were used in two recent studies in China to treat patients with COVID-19 pneumonia. A case report of a critically ill COVID-19 patient on a ventilator was one among the studies and showed evidence of liver injury despite receiving intensive therapy. Treatment was done with allogeneic human umbilical cord MSC as an intravenous infusion every three days (each time 5×10^7 cells) three times. The patient was off the ventilator and able to walk after the second administration. The T cell counts and other measured parameters returned to normal levels, and the injured tissue was repaired with no noticeable side effects [127]. Another study was conducted to examine whether MSC transplantation could improve the consequence in patients with COVID-19 pneumonia. In this study, a single dose of 1×10^6 cells per kilogram of weight were administered. As an assessment period of 14 days, post-MSC injection included monitoring the inflammatory and immune function changes and the overall clinical outcomes. MSCs cured or considerably improved the functional outcomes in patients with no adverse effects. It was observed that the pulmonary function and symptoms improved significantly within 2 d of MSC transplantation. At the end of the treatment period, enhancement in overall markers was detected, this was manifested as an increase in peripheral lymphocytes, decrease in C-reactive protein and also a reduction in the overactivated cytokine-secreting immune (CXCR3+CD4+ T and CXCR3+CD8+ T) cells. Moreover, the disappearance of CXCR3+ NK cells was noticed in 3-6 d.

Additionally, a dramatic rise in a group of CD14+CD11c+CD11bmid regulatory DC cell population was detected. Comparing the MSC treatment group with the placebo control group revealed a significant decrease in TNF-α level and an augmentation in

IL-10 levels. Therefore the safety and efficacy of IV administration of MSCs for managing COVID-19 pneumonia were approved [128]. Furthermore, it was found that the MSCs were ACE2 negative, which proves they were free from the virus (Fig. 4), and this makes them beneficial in COVID-19 patients through immunoregulatory function [129].

MSCs could hold and release biologically active moieties named secretome. They can do their function *via* paracrine. These MSC-secretome are composed of proteins like cytokines, growth factors, chemokines, and EVs that are of micro and nano-size. Moreover, it can manage both acute and chronic lung problems since they resemble the parental MSCs. It was found that secretome could activate endogenous stem cells besides progenitor cells, control inflammatory response, suppress apoptosis, trigger angiogenesis, stimulate remodelling of the extracellular matrix, facilitate chemo-attraction and reduce fibrosis [130]. MSC-secretome surpasses the effect of monoclonal antibodies since the former can act on several cytokines. MSC-secretome showed good effectiveness in both levels in the preclinical stage, *in vivo* and *ex vivo* [131]. Following IV administration, secretome showed high stability in the bloodstream and was found distributed with the blood flow until reaching the lungs. Following that, the secretome can penetrate inside tissues offering immune modulation, reducing inflammation, and it can restore the capillary barrier function, and finally enhance bacterial clearance. Comparing both MSCs and secretome, one can reveal that secretome cannot self-replicate, and therefore, it is safer with no probability of tumour induction. Upon IV injection of secretome, a chance for emboli formation is lesser than MSCs because secretome possesses low immunogenicity [132]. MSC-secretome possesses certain technological advantages since it could be

easily stored at a low cost, and it is available as ready to use product suitable for emergency cases.

Both injection and inhalation routes are available options for MSC-secretome administration. However, the inhalation route is more preferable, and it will give rise to fast action and enable using a small amount of active moiety. A clinical trial with the title 'A Pilot clinical study on inhalation of MSCs-Exo treating severe novel coronavirus pneumonia' (NCT04276987) started and is in Phase I stage [133]. This clinical trial aimed at exploring both the safety as well as efficacy of inhaling allogenic adipose mesenchymal stem cells (MSCs-Exo) in managing hospitalized patients severely infected by a novel coronavirus and suffering from pneumonia. Another clinical trial 'A clinical tolerance study on aerosol inhalation of MSCs exosomes in healthy volunteers' (NCT04313647) is in Phase I stage [137]. After completion, this study will evaluate both the safety as well as tolerance of inhaling exosomes that are derived from allogeneic adipose MSCs in healthy volunteers. Experimental studies have proved that both MSCs and their exosomes had a substantial effect in decreasing lung inflammation and different pathological conditions noticed in diverse types of lung injury. IV administration of MSCs can cause clumping or aggregation in the injured microcirculation, then there will be a high risk of oncogenicity and mutagenicity. This risk does not exist in treatment with nebulized MSCs-Exo. An added benefit for MSCs-Exo is their ease of storage for several weeks/months that enable their safe transportation. A clinical trial with the title 'A phase I/II randomized, double-blinded, placebo trial to evaluate the safety and potential efficacy of intravenous infusion of organicell flow for the treatment of moderate to SARS related to COVID-19 infection vs placebo' (NCT04384445) is in Phase II stage [138]. This study aimed at determining both safety and efficacies of IV

infusion of organicell for managing SARS related to COVID-19 infection and compare it to placebo. Infected patients can develop immune activation with cytokine storm syndrome that represents the primary cause of lung damage that might eventually lead to death. However, the lungs and systemic organ damage can be reduced, and also the alveolar function can be protected by suppressing the extreme immune response in a well-timed manner throughout the disease. Organicell flow is a minimally modified acellular product derived from human amniotic fluid (HAF). It contains more than 300 growth factors, cytokines, chemokines and other EVs that are derived from amniotic epithelial and stem cells. The product has a mean concentration of 5.24×10^{11} particles/ml and a mean mode size of 125.2 nm. The existence of exosome associated proteins CD81, CD63 and CD9, in addition to high expression of CD133, was confirmed by surface marker analysis. Thus the Organicell flow demonstrates the therapeutic potential to suppress cytokine activation to decline the severity of COVID-19 infection. In this study, the intervention model is Parallel Assignment. The trial consists of two groups; each group has 10 subjects. The eligible subjects for the study are randomized and double-blinded to one of the two groups: placebo or therapy. The primary outcome measures of this trial are the incidence of any infusion associated with the adverse events and SAE. Some of the secondary outcome measures include cytokine levels, C-reactive protein levels, survival rate and many more. Further, a clinical trial with the title ‘Aerosol inhalation of the exosomes derived from allogenic COVID-19 T cell in the treatment of early-stage novel coronavirus pneumonia’ (NCT04389385) is active but not recruiting and is in the Phase I stage [139]. This clinical trial aims to test the safety and efficacy of inhaled CSTC (COVID-19 specific T-cells) exosomes to treat early-stage novel coronavirus pneumonia. The escalating levels of systemic inflammation cause severity of COVID-

19 that ultimately leads to hyper-inflammatory stage similar to macrophage activation syndrome and finally death. Hence, there is a need for early intervention to prevent respiratory failure, which requires a decline of the viral load. The immune system contains virus-specific T-cells (VSTs) as a defence against disease-causing viruses. Specific COVID-19 T-cells from the donor are *activated in vitro* and are expanded by exposing them to fragments of peptide and cytokines. The secretion of potent mediators such as IFN gamma in the form of exosomes was further stimulated. This represented a treatment option for controlling disease progression in COVID-19 patients at their early stages of lung disease. This biological agent can be used without a condition for HLA (human leukocyte antigen) match. The primary outcome measures of this trial are AE and SAE, efficacy assessment, as well as recovery rate without a mechanical ventilator for a time frame of 28 d.

Although many studies have been developed both *in vitro* and *in vivo* about exosome, including their use for different treatment and diagnostic purposes, the clinical utility of exosomes needs more studies. Herein, a summary of the factors that might hinder the clinical applications of exosomes will be discussed. For example, it is very critical to choose the right source of exosomes for clinical use as anti-carcinogenic. The cellular origin of exosomes might impact its efficiency when used therapeutically, so more studies have to be performed to compare the results of exosomes obtained from different human cells. Furthermore, many factors might affect exosomal miRNAs' expression level; this includes the stage of tumor and its type. For this reason, diverse clinical trials have to be performed, including several types of cancer and different stages in each type. Another factor that might hinder the application of exosomes in clinical treatment is the absence of standardized techniques for their separation and detection, which put more responsibility on the shoulder of researchers to make

available standardized techniques for ensuring the wide usage of exosomes clinically. Development in stable inner reference genes is still under study; this will ensure more accurate exosomal miRNAs quantification. The isolation and production of a large amount of exosomes are also a major burden. The available methods for exosomes isolation are costly and time-consuming. Moreover, the isolation is not easy enough, and contamination by particles of the same size as exosomes could occur. Furthermore, developing the right dosage form for exosomes is very challenging to ensure their effectiveness in managing different types of cancer. Entrapping miRNAs in exosomes must be high enough in order to produce effective targeted cancer therapy. Cell-specific receptors for each tumor must be well identified to prepare the right targeted miRNAs exosomal preparation. This will help to diminish the off-target effects [140,141]. After choosing the suitable dosage form and preparing it, large-scale studies are needed to approve exosome usage to introduce the risk for both immunosuppression and tumorigenesis [142, 143].

The literature data available related to the use of exosomes in viral infection treatment, especially COVID-19, is practically at the experimental stage. Even though many studies have interesting clinical implications, there are only a few registered clinical trials related to the use of exosomes in the treatment of COVID-19. Thus, there is still much work to be done in this area, including the optimizing and standardization of exosomes.

10. Conclusion

The urgent need arises about treating COVID-19 infected patients developing pneumonia that necessitates exploring new strategies and novel delivery systems. A therapeutic strategy “without cells” is an emerging field having several advantages. Hence, exosomes derived from MSCs and those derived from COVID-19 specific T

cells could be one of the best therapeutic approaches. Clinical trials have been started, and the results are still awaited. It was found that delivering MSCs-Exo rather than live MSCs is safer; however, both significantly reduced lung inflammation and diminished other pathological conditions of various types of lung injury. The continuous and detailed study on the mechanism of viral proteins loading onto exosomes and their roles in managing viral diseases will provide more specific insights on developing exosomes as novel vaccine and drug delivery systems.

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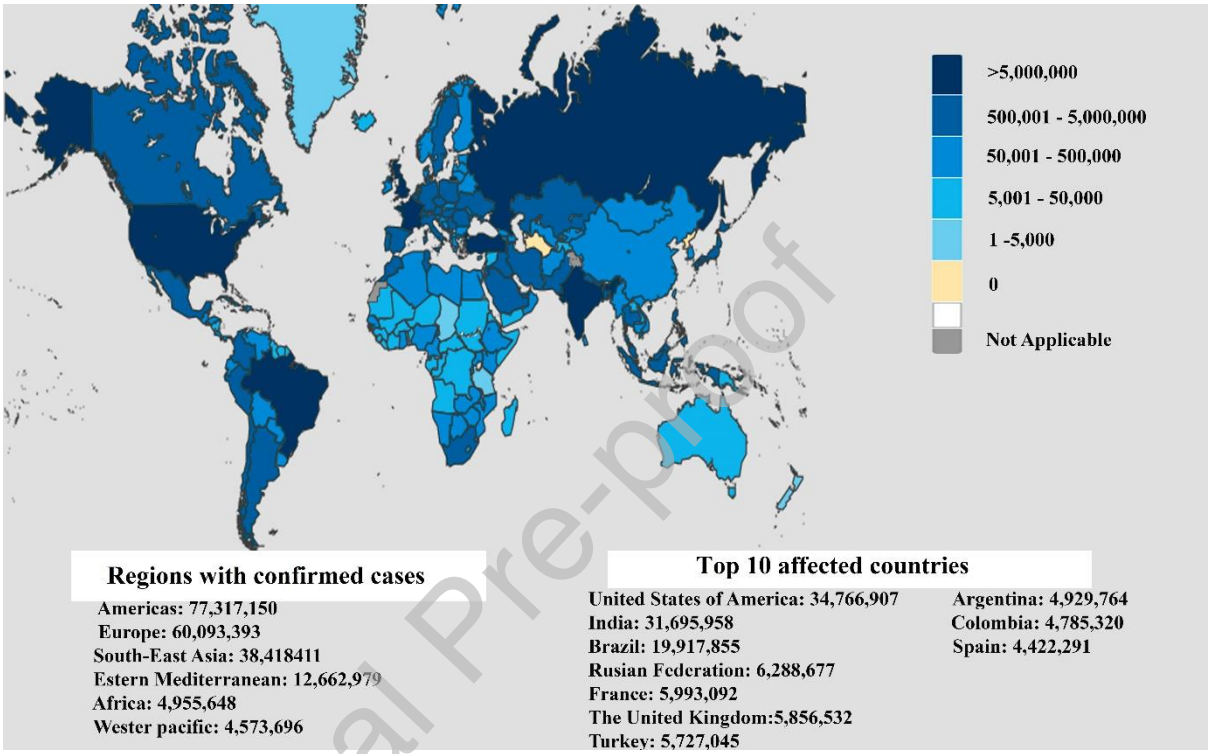

Fig. 1. Map of COVID-19 affected countries reported to WHO as of 2 August 2021.

* Cases – Counts: The ranges from deep blue to light shades as follows: > 1 000 000/500 001 – 1 000 000/50 001 – 500 000/5 001 – 50 000/1 – 5 000

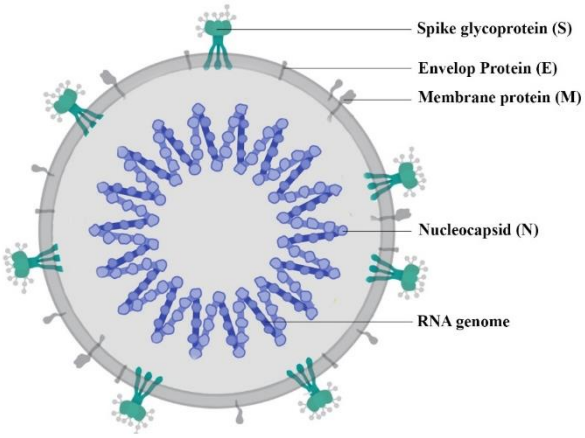

Fig. 2. Structure of SARS-CoV-2

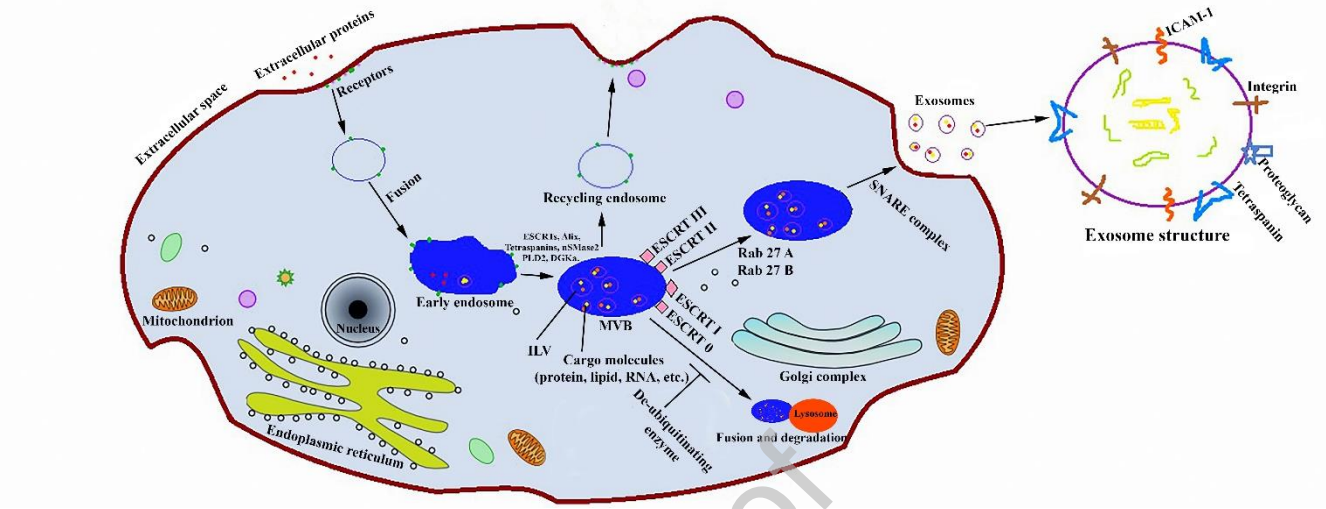

Fig. 3. Biogenesis and structure of Exosomes.

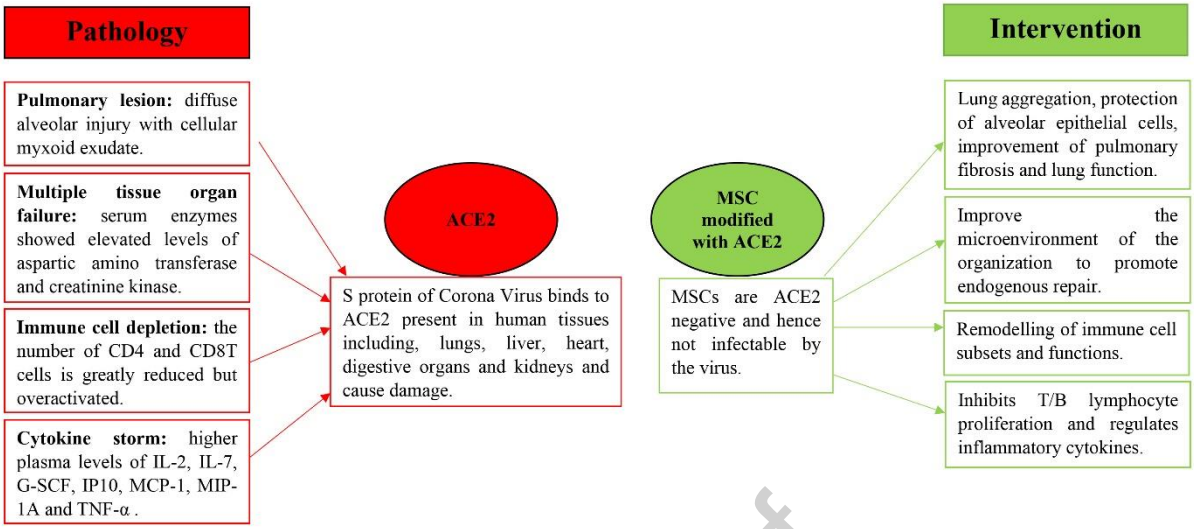

Fig. 4. Benefits of MSC-ACE2 in COVID-19 patients *via* immunoregulatory function.

Table 1 Exosomes facilitate the viral infection

Disease and mode of spread	Virus	Ref.
HIV-1-infected cells release exosomes that inactivate $CD4^+$ T-cells to achieve replication of HIV-1 *via* both a Nef- and ADAM17-dependent mechanism.	HIV	[56]
Exosomes can uptake transactivating response (TAR) RNA, this will aid downregulation and apoptosis and therefore contribute to HIV infection.	HIV	[57]
The apoptosis of T-cell was found to increase due to exosomal Nef leading to depletion of $CD4^+$ T-cell in case of AIDS.	HIV	[58]
Nef supports HIV-1 infection by decreasing the HIV expression of CD4 in exosomes derived from infected cells.	HIV	[59]
Tropical spastic paraparesis is caused by HTLV-1 infection. HTLV-1 infected cell lines released exosomes comprising Tax, a pleiotropic transactivating protein intricated in immune dysregulation linked through infection.	HTLV-1	[60]
HSV-1 infection releases a variety of microvesicles from cells; L particles are most prominent. L particles are made up of virus envelope as well as tegument and are devoid of viral genome as well as capsid proteins. As such they are non-infectious but have been shown to increase the susceptibility for infection in uninfected cells.	HSV-1	[61]
Latent membrane protein 1 which is a signal transduction protein was found in exosomes isolated from EBV^+ cancer cells. The uptake of LMP^+ exosomes inhibited the activity of natural killer cell as well as T-cell activation and proliferation.	EBV	[62]
Exosomes released from EBV^+ cells contained Galectin-9 which induces EBV-specific T-cell apoptosis and hence circumvents detection *via* the immune system.	EBV	[63]
EBV was found on packaging viral miRNAs into exosomes and the miRNAs can decrease CXCL11, a targeted immunoregulatory gene essential for antiviral activity.	EBV	[64]
CMV infection increases DC-SIGN release on exosomes, which belongs to the C-type lectin family and is crucial for uptake of virus. This mediates myeloid DCs infection by CMV and augmented total CMV infectivity.	CMV	[65]
HHV-6 infection increases MHCI transfer to released exosomes, and downregulation of MHCI is a renowned pathway for immunoevasion.	HHV-6	[66]
The presence of viral RNA and proteins in the extracted exosomes out of infected cells with RVFV, led to apoptosis of immune cells exposed to these exosomes.	RVFV	[67]
A phelebovirus incorporated virions into $CD63^+$ exosomes that lead to receptor-independent uptake by neighbouring cells.	SFTSV	[68]

Human immunodeficiency virus (HIV);Human T-cell lymphotropic virus type 1 (HTLV-1);Rift Valley fever virus (RVFV);Herpes simplex virus type 1 (HSV-1);Epstein-Barr virus (EBV);Cytomegalovirus (CMV);Human herpesvirus type 6 (HHV-6);Severe fever with thrombocytopenia syndrome virus (SFTSV); Dendritic cell-specific ICAM3-grabbing-nonintegrin (DC-SIGN); Major histocompatibility complex (MHCI)

Table 2: Antiviral response of exosomes

Antiviral response in disease	Virus	Ref.
HSV-1 incorporates STING protein into exosomes, and delivers it to uninfected cells. The viral miRNAs such as miR-H3, miR-H5 and miR-H6 were also packaged into exosomes, and these exosomes may negatively affect both the host-host infection and viral spread thereby increasing host survival.	HSV-1	[69]
APOBEC3G, cGAMP, miRNA-99, and miRNA-88 incorporated into the exosomes exhibited antiviral effect.	HIV-1	[70-72]
The dUTPase was found to be incorporated into exosomes which produced an antiviral effect.	EBV	[73]
Viral miRNAs and mitochondrial DNA loaded into exosomes exhibited an antiviral effect.	KSHV	[74, 75]
IFI16 and Glycoprotein B were present in the exosomes, which produced an antiviral effect.	CMV	[76, 77]
The extracellular IFITM3 protein present in exosomes was found to contribute to inhibitory effect of DENV entry in cell models of DENV-2 infection *via* interferon-induced inhibition.	DENV	[78]
Exosomes increase the functioning of macrophages and NK cells and deliver antiviral molecules between cells.	HBV	[79]
During influenza virus infection the released exosomes in the airways elicit inflammatory responses in lungs and convey viral antigen that could be exploited by antigen-presenting cells in order to induce a cellular immune response. In addition, the attachment factors α2,3 and α2,6-linked sialic acids that are present on the airway exosomal surface, and can neutralize influenza virus. Thus the virus is unable to bind and enter the target cells.	Influenza virus	[80]

Herpes simplex virus type 1 (HSV-1);Stimulator of INF genes (STING);Apolipoprotein B mRNA editing enzyme, catalytic polypeptide- like 3G (APOBEC3G); Interferon-inducible transmembrane 3 (IFITM3);Human immunodeficiency virus type 1 (HIV-1)Epstein-Barr virus (EBV);Kaposi's sarcoma-associated herpesvirus (KSHV);Cytomegalovirus (CMV);Dengue virus (DENV);Hepatitis B virus (HBV)

Table 3: State-of-the-art literature reviewing of the research studies and clinical trials about mesenchymal stem cells or mesenchymal stem cells derived exosomes for managing COVID-19.

Study/Title	Exosome (Route)	Outcome	Ref.
Aerosol inhalation of MSCs-Exo in managing severe novel coronavirus pneumonia is being explored in a single-arm, open- label, combined interventional clinical trial.	MSCs-Exo (Inhalation)	Ongoing Clinical trial NCT04276987 (China)	[126]
A critically ill COVID-19 patient on treatment with hUCMSCs developed clinical remission.	hUCMSCs (IV)	The laboratory indices and CT images indicated decrease in inflammation symptom. Patients were shifted out of ICU, and the throat swabs tested negative 4 d later. These results showed the clinical outcome and good tolerance of allogenic hUCMSCs transfer.	[127]
Patients with COVID-19 pneumonia benefit from ACE2-MSC transplantation.	ACE2-MSC (IV)	After 2 d of MSC transplantation, these 7 patients had considerably improved pulmonary function and symptoms. It was found to be safe and efficacious in patients with COVID-19 pneumonia, who had been in a critical condition.	[128]
A Phase I/II randomised, double-blind, placebo-controlled trial to assess the safety and potential efficacy of an IV infusion of Zofin (Organicell Flow) to treat moderate COVID-19 infection caused by SARS.	Cytokines, Growth factors, and other EVs/nanoparticles derived from HAF (IV)	Ongoing Clinical trial NCT04384445 (USA)	[129]
A single-arm, open label, combined interventional (phase I/II trials) clinical trial is being conducted to determine the safety and efficacy of inhaled CSTC-exosomes in the treatment of early stage novel coronavirus pneumonia.	CSTC-Exo (Inhalation)	Ongoing Clinical trial NCT04389385 (Turkey)	[130]
Exosomes (ExoFlo^{TM}) derived from allogeneic bone marrow mesenchymal stem cells were used for treating severe COVID-19 in a non-randomized, open-label, cohort study.	Bone marrow MSCs-Exo (IV)	All safety endpoints were met with no adverse events detected. The survival rate was 83%. ExoFlo^{TM} is a promising therapeutic candidate for severe COVID-19 due to its safety profile, capacity to restore oxygenation, downregulate cytokine storm and reconstruct immunity.	[131]

ExoFlo™, bone marrow-derived EVs on IV administration, is being evaluated as a treatment for moderate to severe ARDS in patients with severe COVID-19 in a multi-center, randomized, double-blinded, placebo-controlled clinical trial.	Bone marrow derived EVs (IV)	Ongoing Clinical trial NCT04493242 (USA)	[132]
Exosome inhalation was evaluated for safety and efficacy in SARS-CoV-2 associated pneumonia.	MSCs-Exo (Inhalation)	Ongoing Clinical trial NCT04491240 (Russia)	[133]
To determine the efficacy of MSCs infusion as a supplementary therapy to standard supportive treatment for patients with moderate/severe COVID-19.	MSCs (IV)	Ongoing Clinical trial NCT04444271 (Pakistan)	[134]
hUMSCs and Exosomes for lung injury in patients with COVID-19.	hUMSCs (IV)	Ongoing Clinical trial ChiCTR2000030484 (China)	[135]
CAP-1002 Allogeneic Cardiosphere-Derived Cells	EVs from CDCs (IV)	Ongoing Clinical trial NCT04338347 (USA)	[136]

human umbilical cord mesenchymal stem cells (hUCMSCs);COVID-19 Specific T Cell Derived Exosomes (CSTC-Exo);Acute Respiratory Distress Syndrome (ARDS);Cardiosphere-Derived Cells (CDCs)

Graphical abstract